Urinary levels of regenerating islet-derived protein III β and gelsolin differentiate gentamicin from cisplatin-induced acute kidney injury in rats

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A key aspect for the clinical handling of acute kidney injury is an early diagnosis, for which a new generation of urine biomarkers is currently under development including kidney injury molecule 1 and neutrophil gelatinase-associated lipocalin. A further diagnostic refinement is needed where one specific cause among several potentially nephrotoxic insults can be identified during the administration of multidrug therapies. In this study we identified increases in regenerating islet-derived protein III beta (reg IIIb) and gelsolin as potential differential urinary markers of gentamicin's nephrotoxicity. Indeed, urinary levels of both reg IIIb and gelsolin distinguish between the nephrotoxicity caused by gentamicin from that caused by cisplatin where these markers were not increased by the latter. Reg IIIb was found to be overexpressed in the kidneys of gentamicin-treated rats and excreted into the urine, whereas urinary gelsolin originated from the blood by glomerular filtration. Our results illustrate an etiological diagnosis of acute kidney injury through analysis of urine. Thus, our results raise the possibility of identifying the actual nephrotoxin in critically ill patients who are often treated with several nephrotoxic agents at the same time, thereby providing the potential for tailoring therapy to an individual patient, which is the aim of personalized medicine.

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Gentamicin is an aminoglycoside antibiotic widely used worldwide against Gram-negative infections. Its therapeutic efficacy and use are severely handicapped by its toxicity, which mainly occurs at the renal and auditory levels.[1] Gentamicin-induced nephrotoxicity appears in 10–25% of therapeutic courses.[2–4] It is characterized mostly by tubular damage,[5,6] but glomerular[1,7–11] and vascular[12–14] alterations might also appear in a dose-dependent manner.[15] Tubular damage affects mainly the proximal compartment,[5] and it results in a reduced glomerular filtration rate through (1) an impairment of the reabsorption capacity that activates the tubuloglomerular feedback to prevent massive fluid loss;[16] and (2) an increased intratubular pressure resulting from tubular obstruction by tissue debris.[5] In the glomeruli, gentamicin produces contraction of mesangial cells, which diminishes the ultrafiltration coefficient (K_f).[1,8,17,18] As a polycation, gentamicin also alters the electrical properties of the glomerular filtration barrier (GFB).[19,20] Finally, gentamicin reduces renal blood flow (RBF) by contracting both preglomerular arteries and the afferent and efferent arterioles.[12] As a consequence of a diminished RBF, glomerular filtration rate becomes deteriorated. A lower RBF also contributes to tissue necrosis, especially within the cortical area.[21] Glomerular and vascular contraction is mediated by locally increased autacoids like arachidonic acid derivatives (that is, thromboxane A2; Papanikolaou *et al.*[22]), platelet-activating factor,[6,23] and endothelin-1.[24–26] Vasoconstriction might also be related to deterioration of the relaxing capacity of the vascular smooth muscle.[14]

Depending on other comorbid conditions, gentamicin-associated nephrotoxicity may lead to an acute renal failure (ARF), an extremely serious condition resulting from an abrupt loss of the kidneys' excretory function sufficient to prevent blood cleansing of waste products, and to impair water and electrolytic balance.[27–29] ARF poses an enormous human and socioeconomic burden derived from its high incidence and mortality rate. It is estimated that nearly 1% of hospital admissions are associated with ARF, and ~2–7% of

hospitalized patients eventually develop ARF.[30,31] Most importantly, mortality among ARF patients stays strikingly high at ~50% of the cases in spite of dialysis instauration.[30–32] This rate grows up to an 80% when ARF courses with multiorgan damage.[30,32,33] The most effective handling of ARF relies on the earliest possible detection, upon which hydration and treatment withdrawal or regime modifications are applied. Other occasional measurements include the preemptive administration of renoprotective drugs, such as the antioxidants *N*-acetylcysteine[34–37] or amifostine,[38] and other molecules presently under development for the prevention of renal injury. Intervention on severe cases relies mostly on dialysis.

In the clinical practice, ARF is diagnosed when renal dysfunction induces an increase in creatinine and urea levels in the blood. However, at this stage, glomerular filtration rate is already decreased and ARF becomes difficult to handle. As such, present diagnostic tendencies aim at detecting incipient pathophysiological events occurring at early stages, when damage is less extensive.[32,39–41] Measurement of urinary enzymes originating from renal tissue injury, including *N*-acetyl-β-D-glucosaminidase (NAG) and others,[40] is presently the finest method for an early detection of ARF coursing with tubular damage. New, early urine markers are currently in an advanced degree of validation for the diagnosis and prognosis of ARF, including kidney injury molecule 1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), and others.[40,42]

The next step in the refinement of ARF diagnosis will be the capability to differentiate the renal damage inflicted by a determined drug or insult from that exerted by others.[43] It could be based on specific collections of markers composing a molecular fingerprint for every potential nephrotoxicant. This new diagnostic capability will be very useful for a more rational, individualized, and specific handling of clinical situations coursing with symptoms of renal damage, as for example, in the case of polymedicated patients receiving cotreatments containing two or more drugs being potentially nephrotoxic. This will allow us to correctly redirect the treatment by substituting only the harmful drug or by reshaping its therapeutic regime. In this article, we identify regenerating islet-derived protein III β (reg IIIb) and gelsolin as new urinary markers to further profile and differentiate the AKI inflicted by gentamicin from that induced by cisplatin.

RESULTS

Characterization of the renal lesion induced by gentamicin

As expected, after 6 days of treatment, gentamicin caused a marked ARF with an associated mortality of ~50% (Figure 1b). Surviving animals coursed with a small but significant weight loss and polyuria. ARF was further characterized by a dramatic increase in plasma creatinine and urea concentration, suggesting a reduction of glomerular filtration rate (Figure 1a). NAG (Figure 1a), KIM-1, and bone morphogenetic protein 7 (Figure 1c) urinary excretion also increased, indicating tubular damage. Proteinuria was also evident in the urine of animals treated with gentamicin (Figure 1a). Hematoxylin–eosin-stained renal sections (Figure 1d) revealed a clear tubular necrosis in gentamicin-treated rats. No gross modification of the glomeruli was evident. At the papillary level, obstruction of collecting tubuli with hyaline material was widespread in gentamicin-treated animals.

Differential proteomic analysis of the urine

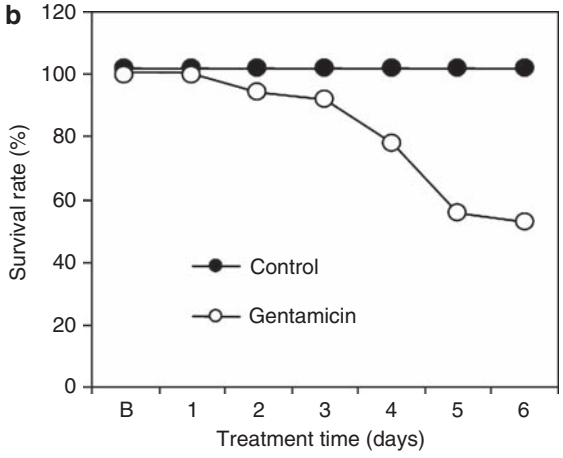
A representative image of two-dimensional (2D) gels (pH range 4–7) of urine samples from control and gentamicin-treated rats is shown in the upper panels of Figure 2. Many proteins concentrate in the range of pH 4.5–5.5. For that reason, 2D separations in this pH range were also done with the same urine samples. A representative image of the latter is shown in the lower panels of Figure 2. A great similarity was observed between samples from animals in the same group, and high reproducibility was obtained when repeating the 2D separation with the same sample, for quality assurance. However, the urine proteome of both groups is substantially different. Statistically significant, differentially present spots between control and gentamicin groups were recognized and numbered for chemical identification. Mass spectrometric analysis revealed the identity of two proteins increased in the urine of gentamicin-treated rats, which showed potential interest after discarding most of the other proteins, normally found in different proteinuric conditions. They were identified as reg IIIb and gelsolin (Figure 2).

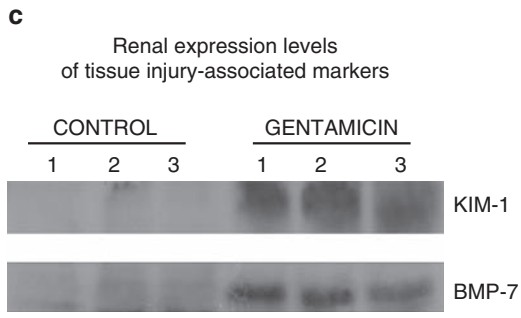
Reg IIIb and gelsolin are differentially excreted in the urine of rats treated with gentamicin

The increased urinary level of these proteins in the urine of gentamicin-treated rats was confirmed by western blot analysis. Moreover, the urine from rats treated with a nephrotoxic regime of cisplatin was also analyzed. Figure 3b shows data on plasma creatinine concentration and blood urea nitrogen from 6 control rats, 6 rats treated with gentamicin, and 6 rats treated with cisplatin. It demonstrates that animals treated with gentamicin or cisplatin developed an overt renal failure as demonstrated by the increment in plasma blood urea nitrogen and creatinine, increased NAG excretion, and the decrease in creatinine clearance. These urine samples were also analyzed for their content in reg IIIb and gelsolin. Figure 3a clearly shows that the urinary level of reg IIIb is markedly increased only in gentamicin-treated animals, despite undergoing a similar degree of renal damage than cisplatin-treated rats. Western blot of gelsolin revealed two reactive bands. The higher one corresponds to the full-length protein, whereas the lower one corresponds to a fragment thereof. The presence of gelsolin within the reactive bands was further re-confirmed by tandem mass spectra. Treatment with gentamicin induces the appearance in the urine of both the full-length gelsolin and the ~43 kDa fragment. However, the full-length band was absent in the

a

	Δ Weight (%)	Plasma creatinine (mg/dl)	Cl_{cr} (ml/min)	Plasma urea (mg/dl)	Protein excretion (mg/day)	NAG excretion (AU/day)	Urinary flow (ml/day)
Control	105 ± 4	<0.5	≥0.93 ± 0.15	45.9 ± 1.9	7.3 ± 0.9	0.54 ± 0.11	10 ± 1
Gentamicin	95 ± 3 [a]	3.3 ± 0.4 [a]	0.17 ± 0.06 [a]	435.8 ± 47.9 [a]	40.0 ± 7.4 [a]	7.44 ± 2.12 [a]	18 ± 3 [a]





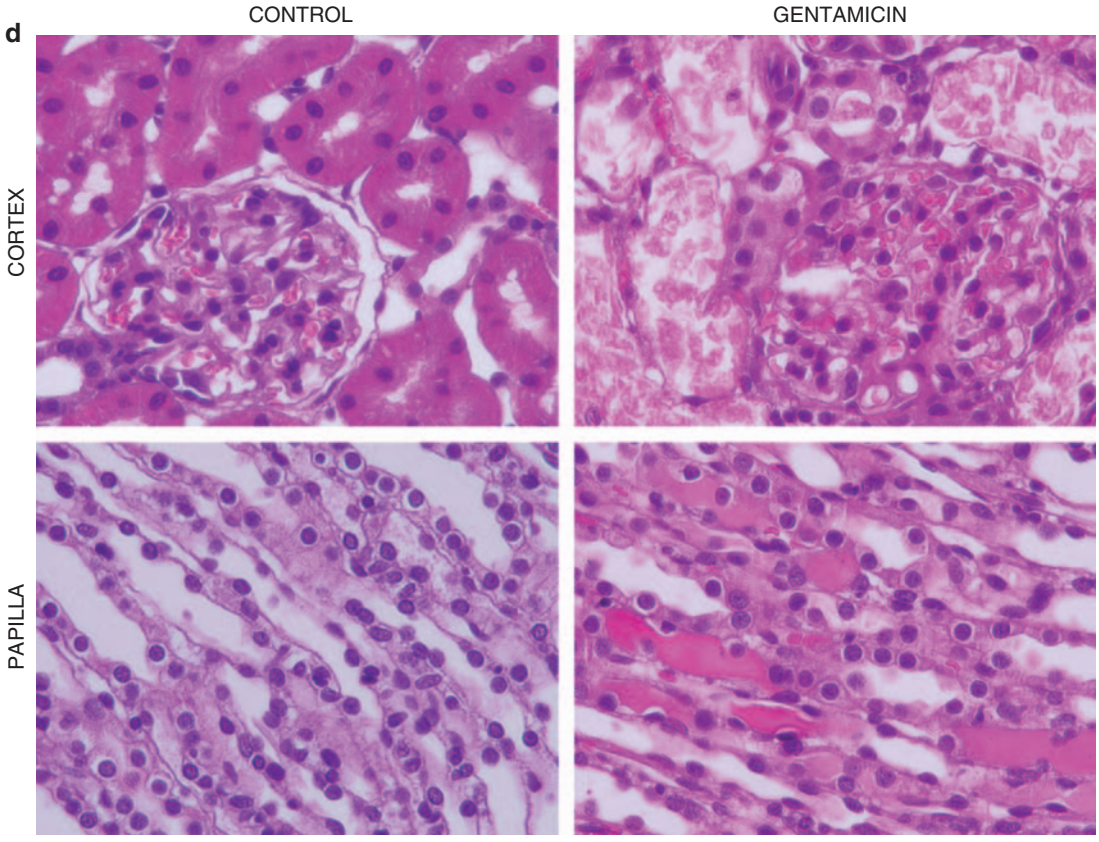
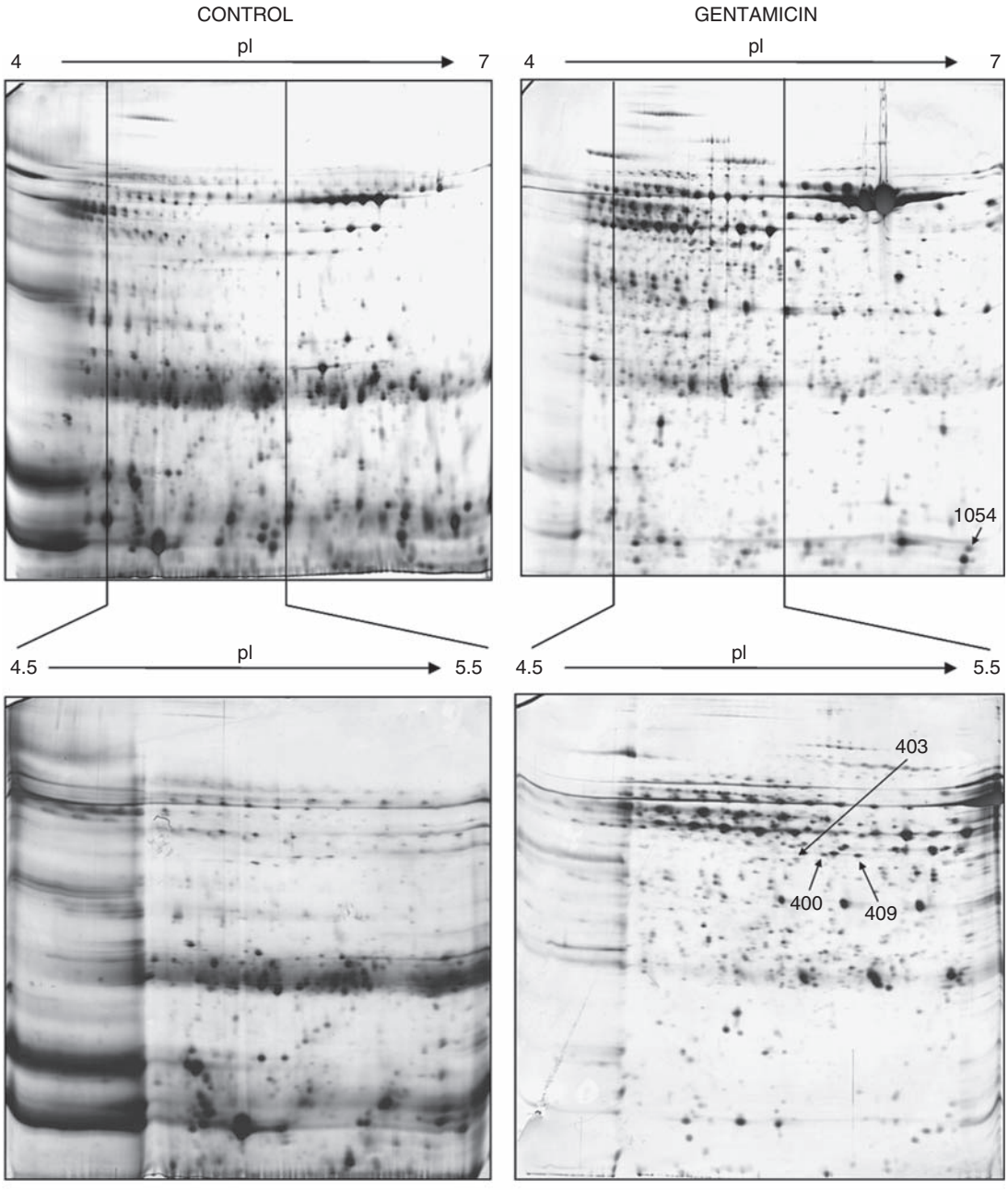


Figure 1 | Characterization of the renal damage induced by gentamicin. (**a**) Body weight (shown as a percentage of the initial weight), plasma creatinine concentration, creatinine clearance (Cl_{Cr}), plasma urea concentration, proteinuria, *N*-acetyl-β-D-glucosaminidase (NAG) excretion, and urine flow parameters of rats from control and gentamicin groups after 6 days of treatment. (**b**) Survival rate represented as a percentage of surviving animals in each group after 6 days of treatment. (**c**) Representative images of western blot analysis of kidney injury molecule 1 (KIM-1) and bone morphogenetic protein 7 (BMP-7) expression levels in kidney homogenates from 3 randomly selected, control, and 3 gentamicin rats after 6 days of treatment. (**d**) Representative images (original × 1000 magnification) of renal sections stained with hematoxylin and eosin from gentamicin and control rats. Data represent the average ± s.e.m. AU, arbitrary units; B, basal time point; $^{a}P \leqslant 0.05$ with respect to the control group. In both groups, $n = 12$ at the beginning of the experiment.



Protein name	Accession No.	Protein MW (kDa) / pI	Spot No.	Score	No. of peptides	% Sequence coverage
Gelsolin	Q68FP1	86.1/5.75	400	100	3	3
			403	85	3	3
			409	116	3	3
Regenerating islet-derived protein 3 β	P25031	20.0 / 7.56	1054	107	7	46

Figure 2 | Urinary proteomics. Two-dimensional (2D) gel images of differentially expressed regenerating islet-derived protein III β (reg IIIb) and gelsolin. Spots were subject to quantitative intensity analysis, labeled with numbers corresponding to those in table, and identified by liquid chromatography-electrospray ionization-quadrupole time-of-flight (LC-ESI-Q-TOF) mass spectrometry. Each gel shown in this figure is representative of eight gels obtained with urine from four randomly selected animals in each group, each one analyzed in duplicate.

urine of all rats treated with cisplatin, except for one of them. Extensive analysis of the urine from other cisplatin-treated rats shows no presence of the full-length band (data not shown).

Time course evolution of reg IIIb and gelsolin urinary excretion in rats treated with gentamicin or cisplatin

We further analyzed the time course evolution of the urinary excretion of these proteins in rats treated with gentamicin or

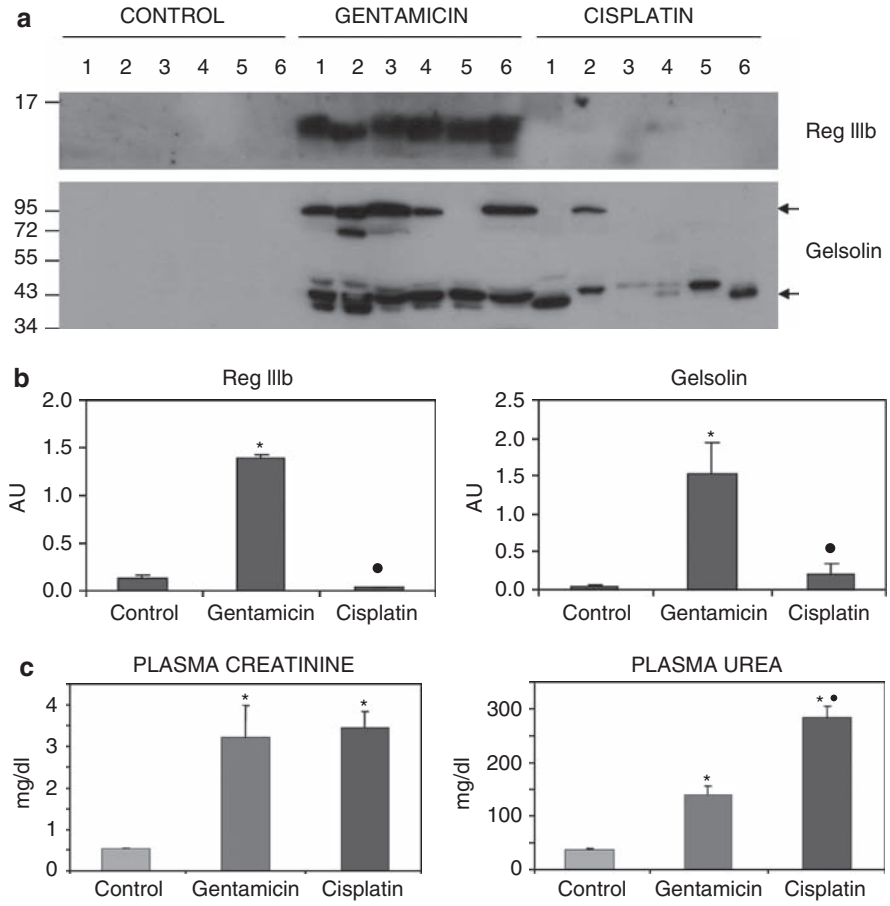


Figure 3 | Validation of the increased urinary excretion of regenerating islet-derived protein III β (reg IIIb) and gelsolin. (**a**) Western blot analysis of reg IIIb and gelsolin in the urine of six randomly selected rats treated with vehicle (control), gentamicin, or cisplatin. Arrows indicate the full-length and t-gelsolin fragment. (**b**) Densitometric quantification of the reg IIIb and full-length gelsolin bands from **a**. (**c**) Plasma creatinine and urea concentration of rats as in **a**. Data represent the average ± s.e.m. AU, arbitrary units; *$P<0.05$ vs control; •$P<0.05$ vs gentamicin.

cisplatin. Figure 4 shows the temporal profile of the renal damage inflicted by gentamicin. Significant damage only occurs after 4 days of treatment, as revealed by the evolution of plasma creatinine, NAG excretion, proteinuria, and the urinary level of three sensitive markers of kidney injury, such as KIM-1, NGAL, and PAI-1 (plasminogen activator inhibitor 1). Congruently with the accumulated knowledge,[44] plasma creatinine is the least sensitive of all the markers tested. Furthermore, histological analysis of renal sections after 3 days of treatment reveals no findings of tubular damage. At this time point, cytoplasmic vacuolation of tubule epithelial cells is evident (Figure 4d), probably resulting from the reported accumulation of gentamicin in the endosomal compartment,[45,46] and from alteration of the endocytic pathway and the endosomal trafficking.[47,48] In this scenario, western blot analysis showed that reg IIIb appears in the urine along with most other sensitive markers of renal injury, starting on day 4. Interestingly, urinary gelsolin (the ~43 kDa fragment) appears as early as on day 1 and stays high through the treatment, long before all other sensitive markers do, including KIM-1, PAI-1, NGAL, and NAG. In the case of rats treated with cisplatin, reg IIIb and full-length gelsolin are not significantly increased in the urine during the same period of 6 days (Figure 5a), despite that rats treated with cisplatin develop a similar degree of renal injury to that observed in gentamicin-treated rats. This is evidenced by the evolution of parameters of renal dysfunction and damage, such as plasma creatinine, urinary excretion of NAG and KIM-1, and by the histological examination of renal tissue after 6 days of treatment with both drugs (Figure 5a–c, and also Figures 3 and 4). Yet, the level of the 43 kDa fragment of gelsolin increases in the urine of rats treated with cisplatin more pronouncedly than and before KIM-1 (Figure 5a), which also occurs in the case of the treatment with gentamicin (Figure 4). This can be potentially exploited for an early diagnosis of AKI.

Origin of urinary reg IIIb and gelsolin

Western blot analysis of albumin-depleted plasma from control and gentamicin-treated rats indicated that reg IIIb is absent (to the detection limit of this technique), whereas gelsolin is normally found in the blood compartment. Even more, gentamicin treatment slightly increases the plasmatic level of the latter (Figure 6a). Gene expression analysis

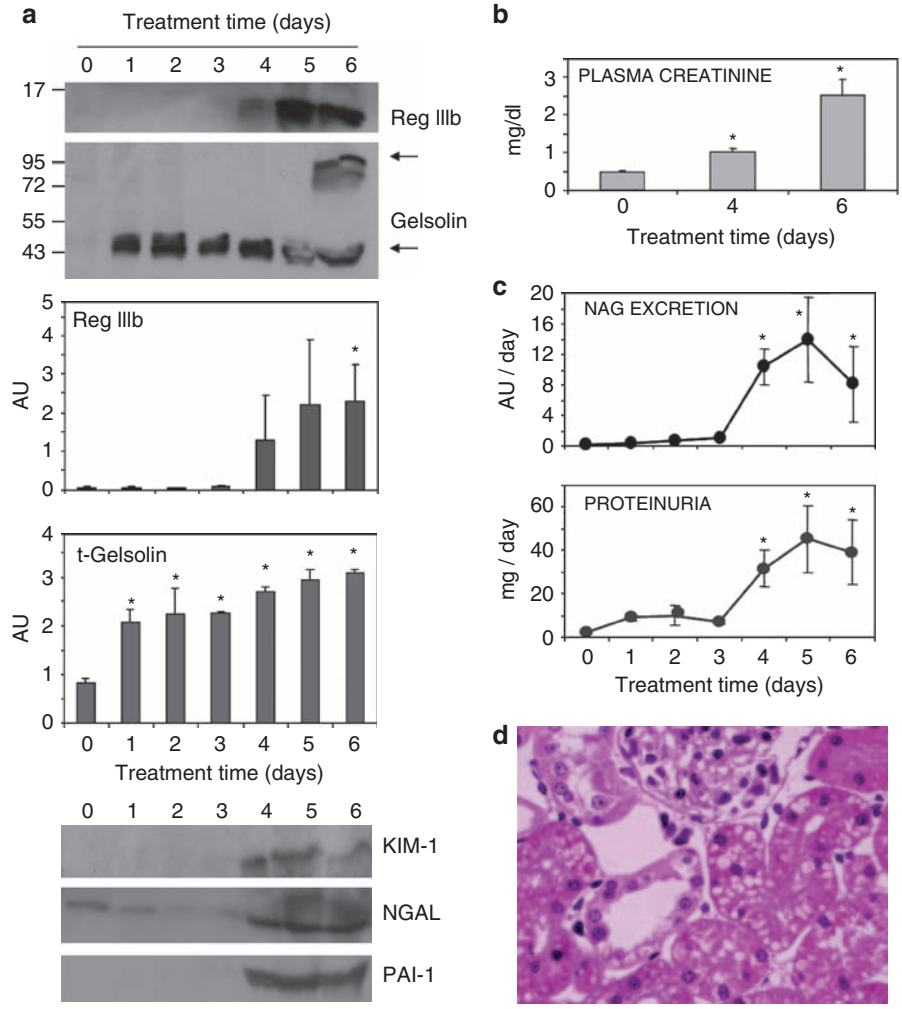


Figure 4 | Time course evolution of urinary regenerating islet-derived protein III β (reg IIIb) and gelsolin in rats treated with gentamicin. (**a**) Representative images of western blot analysis of urinary reg IIIb and gelsolin; densitometric quantification of the reg IIIb and t-gelsolin bands from three western blots carried out with the urine of three unselected rats; and western blot analysis of kidney injury molecule 1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), and plasminogen activator inhibitor 1 (PAI-1) evolution. (**b**) Plasma creatinine concentration. (**c**) Evolution of *N*-acetyl-β-D-glucosaminidase (NAG) excretion and proteinuria of rats treated with gentamicin. (**d**) Representative image of renal sections stained with hematoxylin and eosin from rats treated with gentamicin for 3 days (original magnification ×1000). In all experiments, $n = 3$. Data represent the average ± s.e.m. AU, arbitrary units; $^*P < 0.05$ vs time 0.

carried out on renal tissue by reverse transcriptase-PCR showed that these two proteins are normally expressed in the kidneys. Treatment of rats with gentamicin does not modify the renal expression pattern of gelsolin, but induces an increase in reg IIIb gene expression as early as on day 3 (Figure 6b), when no detectable kidney injury has occurred yet (Figure 4). On day 6, reg IIIb expression is highest.

In order to study whether the origin of these urinary proteins was the blood, which would shed them to the urine through the GFB, we perfused the kidneys of rats treated for 6 days with gentamicin with Krebs solution (containing dextran to compensate for the oncotic pressure). We found that immediately before substituting the renal blood flow with Krebs, we could still detect reg IIIb and gelsolin in the urine (Figure 6c). However, once the renal blood flow was substituted with Krebs flow, gelsolin disappeared from the urine; yet, the upper band of reg IIIb was still detected, whereas the lower one disappeared.

DISCUSSION

Nephrotoxicity poses a considerable health and economic problem worldwide. It is an important reason of failure along the drug discovery process, which leads to discarding otherwise clinically interesting molecules.[49,50] Most importantly, ~25% of the 100 most used drugs in intensive care units are potentially nephrotoxic.[51] Overall, it is estimated that nephrotoxicity is responsible for 10–20% of the acute renal failure cases.[52] A critical aspect for the optimal clinical handling of AKI is an early diagnosis.[53,54] Important progress has been made in the last decade toward an increasingly earlier detection based on novel and more sensitive urinary

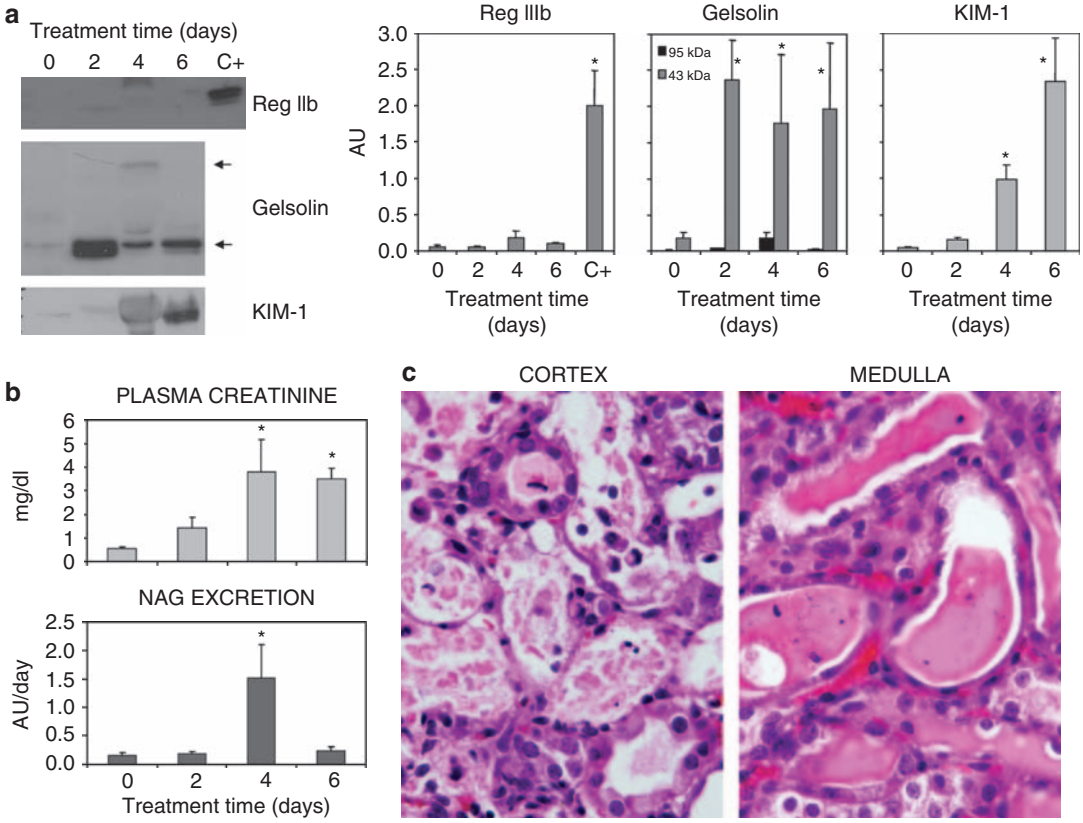


Figure 5 | Time course evolution of urinary regenerating islet-derived protein III β (reg IIIb) and gelsolin in rats treated with cisplatin. (**a**) Representative images of western blot analysis of urinary reg IIIb, gelsolin (full-length and the t fragment, indicated by the arrows), and kidney injury molecule 1 (KIM-1), and densitometric quantification of three independent experiments. (**b**) Evolution of plasma creatinine concentration and *N*-acetyl-β-D-glucosaminidase (NAG) excretion; $n = 6$. (**c**) Representative images of the cortex and medulla in renal sections stained with hematoxylin and eosin from rats treated with cisplatin (day 6 after cisplatin injection; original magnification $\times 1000$; $n = 3$). Data represent the average ± s.e.m. *$P < 0.05$ vs time 0; AU, arbitrary units; C+, positive control (that is, urine from a gentamicin-treated, nephrotoxic rat).

markers.[40] However, AKI diagnosis may still be improved in an individual-drug basis, for enhanced theranostics and a more individualized medicine. In this article we provide some evidence on new urinary markers with a potential to differentiate the nephrotoxicity of gentamicin from that caused by cisplatin, and to detect the renal effects of gentamicin earlier with state-of-the-art AKI markers. They will help to better delineate the pharmacological profile of gentamicin and, in turn, to improve its clinical utility.

Both reg IIIb and full-length gelsolin have potential for a differential or etiological diagnosis of gentamicin's nephrotoxicity. They appear in the urine of rats with overt renal failure induced by gentamicin, but are not present in the urine of rats with a similar degree of renal damage inflicted by cisplatin. Reg IIIb is a 17 kDa member of the calcium-dependent lectin (C-type lectin) superfamily[55] comprising several secretory protein products of four genes (*Reg I*, *II*, *III*, *and IV*). *Reg* genes have been found in different mammal species including human, rat, and mouse. Rat *Reg* genes map to the 4q33–q34 chromosomal region.[56] In humans, all *Reg* genes, except *Reg IV*, map to the 2p12 region.[57] In general terms, Reg family proteins are involved in tissue regeneration in a number of physiological and pathological situations, most prominently including pancreatitis, and also hepatic injury, diabetes, and cancer.[55] Our experiments suggest that reg IIIb may be implicated in renal tissue injury and repair during gentamicin treatment and, importantly, that it might be used as a differential urinary marker. Because we could not detect this protein in the blood, we thought that urinary reg IIIb may be originated in the renal tissue. Indeed, our data indicate that reg IIIb expression is strongly induced by gentamicin in the kidneys, even preceding urine and plasmatic markers and histological findings of nephrotoxicity (on day 3 of treatment; Figure 5b). The renal origin of reg IIIb upon treatment with gentamicin is further supported by our experiments on renal perfusion. When we acutely substituted the RBF for perfused Krebs solution in rats previously treated with gentamicin, we still observed reg IIIb in the urine (Figure 5c). Urinary reg IIIb appears as a double band in western blot analysis, corresponding to a double spot in 2D gels. However, when blood is substituted for Krebs in the renal circuit of gentamicin-treated rats, only the upper band is detected in the urine. We can only speculate that the lower band corresponds to a proteolytic fragment produced

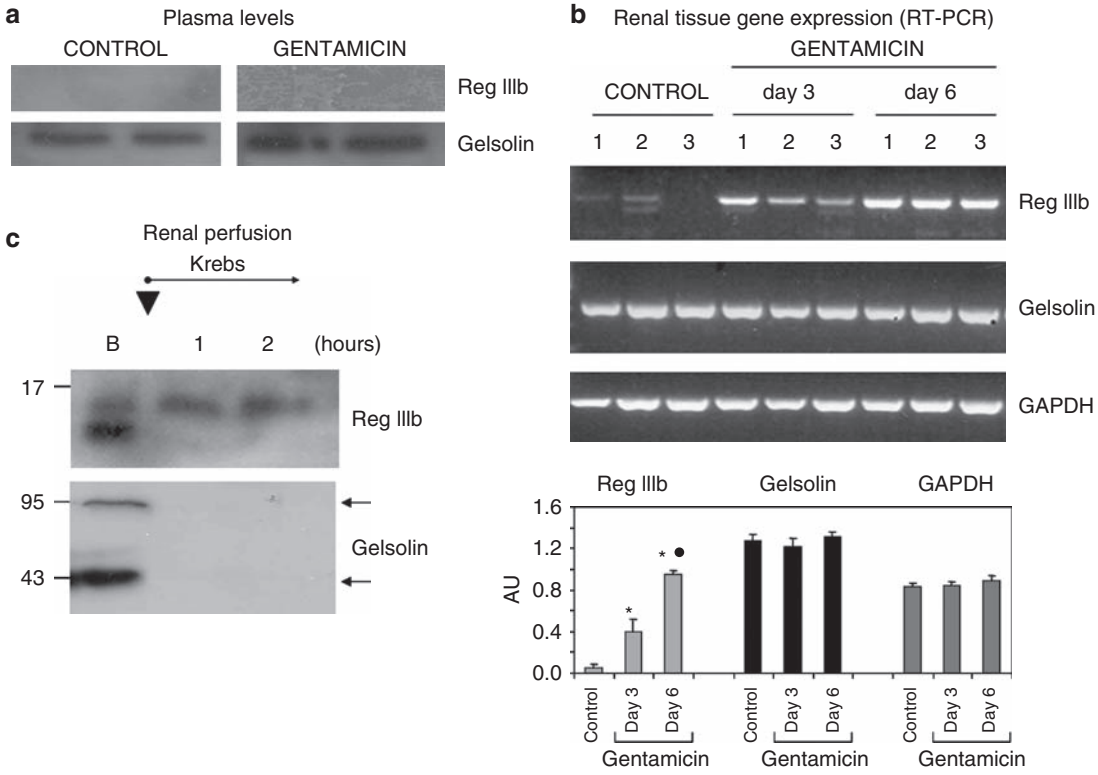


Figure 6 | Origin of urinary regenerating islet-derived protein III β (reg IIIb) and gelsolin. (**a**) Representative images of western blot analysis of the plasma level of reg IIIb and gelsolin from two randomly selected rats treated with vehicle (control) or gentamicin for 6 days. (**b**) Renal tissue reg IIIb, gelsolin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression by reverse transcriptase-PCR (RT-PCR) from three randomly selected rats treated with vehicle (control) or gentamicin for 3 and 6 days; and densitometric quantification of RT-PCR bands. AU, arbitrary units. (**c**) Renal perfusion experiments. Representative mages of western blot analysis of the urinary level of reg IIIb and gelsolin from rats treated with gentamicin for 6 days, and then subject to renal perfusion with Krebs solution, immediately before the beginning of perfusion (B) and 1 and 2 h during perfusion; $n = 3$. Data represent the average ± s.e.m. $^{*}P < 0.05$ vs control; $^{\bullet}P < 0.05$ vs day 3.

by serum proteases, or renal proteases activated by serum components.

Gelsolin is a highly conserved 82 kDa protein involved in cytoskeleton organization and rearrangement in a number of normal cellular processes including motility, signaling, and apoptosis,[58] and pathophysiological conditions such as inflammation, cancer, and amyloidosis.[59,60] Gelsolin is expressed in many cell types and is also secreted and found normally in the blood of vertebrates.[60,61] Gelsolin is a known substrate for effector caspase 3, which yields a 42 kDa proteolytic fragment (t-gelsolin[62]) involved in the execution[63] and regulation[58,62,64] of apoptosis. Our results indicate that urinary gelsolin may also be developed as a marker for the differential diagnosis of gentamicin's nephrotoxicity. In fact, the band corresponding to the full-length protein in western blot studies appears in the urine of gentamicin-treated rats, but it is mostly absent in cisplatin-treated rats. On the contrary, the ~43 kDa band in our gels, likely t-gelsolin, is common to both gentamicin and cisplatin groups. The results shown in Figure 5 indicate that gelsolin gene expression is not modified in the kidneys of rats treated with gentamicin (with respect to controls). They further show that gelsolin disappears from the urine when RBF is substituted for Krebs, suggesting that urinary gelsolin is probably filtered from the blood through the GFB. This may also explain why the full-length gelsolin is detected in the urine of rats treated with gentamicin and not in those treated with cisplatin, whereas t-gelsolin appears after both treatments. Gentamicin alters the GFB properties, leading to an increase in filtration of specific proteins.[19,20] The polycationic charge of gentamicin alters the electrostatic properties of the GFB, increasing the permeability of negatively charged proteins (such as gelsolin, pI = 5.75) and lowering the sieving coefficient of positively charged ones.[65] No alterations of the GFB sieving properties have been reported for cisplatin. In this case, full-length gelsolin would be excluded from passing through the GFB for size restriction.[66,67] However, t-gelsolin would not be trapped in the blood in any case because its lower size allows it to filter more easily through the GFB. In a scenario of tubular necrosis, a larger amount of proteins escapes the handicapped tubular reabsorption capacity, which can be detected in the urine. As shown in Figure 4, t-gelsolin appears in the urine of gentamicin-treated rats significantly earlier than traditional and new AKI markers, the latter including KIM-1, NGAL, NAG, and PAI-1. This might also be exploited for an early monitoring of gentamicin's nephrotoxicity.

The present study provides two novel urinary biomarker candidates for the differential diagnosis of gentamicin's nephrotoxicity, which need to be further developed in the preclinical and clinical settings for a better theranostic usage and efficacy of this drug. This includes the technological development of more practical detection methods for clinical use. Moreover, it poses a proof of principle for the potential application of the etiological diagnosis of AKI to critical patients coursing with multiple conditions potentially affecting renal integrity, including polymedication. Etiological diagnosis should be extended to many other potentially nephrotoxic drugs widely used in the clinical practice, and on pre-and post-renal causes of AKI. This will enable us to delineate patterns of markers that specifically discriminate the origin of undesirable renal effects in order to appropriately and selectively reshape the clinical handling and therapeutic regimes of patients at risk.

MATERIALS AND METHODS

Unless otherwise indicated, all reagents were purchased from Sigma (Madrid, Spain). Gentamicin sulfate was kindly provided by Schering-Plough (Madrid, Spain).

Animals and experimental protocol

Animals were treated in accordance with the Declaration of Helsinki Principles and the Guiding Principles in the Care and Use of Animals stated in the international regulations and in the following European and national institutions: Conseil de l'Europe (published in the Official Daily N. L358/1-358/6, 18 December 1986), Spanish Government (published in Boletín Oficial del Estado N. 67, pp 8509-8512, 18 March 1988, and Boletín Oficial del Estado N. 256, pp 31349-31362, 28 October 1990). Female Wistar rats weighing 200–250 g were allocated under controlled environmental conditions in individual metabolic cages, for 24-h urine samples collection. Rats were randomly divided into three groups: (1) control group (C), receiving daily vehicle intraperitoneal for 6 days; (2) gentamicin group (G), receiving gentamicin intraperitoneal (150 mg/kg body weight per day) for 6 days; and (3) cisplatin group (Cisp), receiving one intraperitoneal dose of cisplatin (7.5 mg/kg). At the end of the experiment in all cases, and at day 3 in some gentamicin-treated rats, kidneys were perfused by the aorta with saline (0.9% NaCl) and immediately dissected. One was frozen in liquid nitrogen and subsequently kept at −80 °C for western blot studies. The other one was fixed in buffered 3.7% p-formaldehyde for histological studies. Blood samples were also obtained in heparinized capillaries at different time points by a small incision in the tail tip. Blood was centrifuged and plasma was kept at −80 °C until use. Urine was cleared by centrifugation, and it was stored at −80 °C until use.

Histological studies

Paraffin blocks were made and 5-μm tissue sections were stained with hematoxylin and eosin. Photographs were taken using an Olympus BX51 microscope connected to an Olympus DP70 color, digital camera (Olympus, Barcelona, Spain).

Biochemical measurements

Plasma and urinary creatinine (Cr_p and Cr_u, respectively) and blood urea concentration were measured using automated analyzer Reflotron (Roche Diagnostics, Barcelona, Spain; lower detection limit of 0.5 mg/dl). Creatinine clearance (Cl_{Cr}) = $Cr_u \times$ 24-h urine output $\times Cr_p^{-1}$. Urine protein concentration was measured using the Bradford method.[68] Urine NAG content was determined by a colorimetric method with a commercial kit (Roche Diagnostics) based on the conversion of 3-cresolsulfonphthaleinyl-*N*-acetyl-β-D-glucosaminide into the purple 3-cresol-cresolsulfonphthaleinyl.

Western blot

Western blots were run with (1) urine samples (21 μl per sample), (2) tissue extracts (100 μg total protein per sample) prepared by homogenizing the kidneys with a tissue mixer (Ultra-Turrax T8, IKA-Werwe; IKA, Staufen, Germany) at 4 °C in homogenization buffer (140 mmol/l NaCl, 20 mmol/l Tris-HCl pH = 7.5, 0.5 M EDTA, 10% glycerol, 1% Igepal CA-630, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 mmol/l phenylmethylsulphonyl fluoride), or (3) albumin-free blood plasma. Albumin was removed from plasma with a column-based, commercial kit based on the immunological retention of rat albumin (Qproteome Murine Albumin Depletion Kit; Quiagen, Madrid, Spain). Samples were separated by electrophoresis in 10–15% acrylamide gels (Mini Protean II system; Bio-Rad, Madrid, Spain). Immediately, proteins were electrically transferred to an Immobilon-P membrane (Millipore, Madrid, Spain). Membranes were probed with antibodies against KIM-1 (R&D Systems, Minneapolis, MN), bone morphogenetic protein 7 (Santa Cruz Biotechnology, Santa Cruz, CA), NGAL (MBL, Woburn, MA), PAI-1 (BD Biosciences, Madrid, Spain), reg IIIb (R&D Systems), and gelsolin (Santa Cruz Biotechnology). The signal obtained was quantified by densitometry using the Scion Image software (Scion Corporation, Frederick, MD).

Two-dimensional electrophoretic separation of proteins

Urine was concentrated and desalted through Amicon Ultra 5K cutoff columns (Millipore). Protein concentration was determined using the Bradford method. For 2D electrophoresis, urine proteins (100 μg) were precipitated with the Clean-Up kit (GE Healthcare, Madrid, Spain). Precipitated proteins were rehydrated in 7 M urea, 2 M thiourea, 4% (w/v) Chaps, 0.5% ampholytes (pH 4–7 or 4.5–5.5), 50 mmol/l dithiothreitol, and bromophenol blue, and isoelectrically focused (500–8000 V) through 18-cm long IPG (immobilized pH gradient) strips, pH 4–7 or 4.5–5.5 (GE Healthcare), using an IPGphor apparatus (GE Healthcare). After focusing, IPG strips were pre-equilibrated for 15 min in equilibration buffer (50 mmol/l Tis-HCl pH = 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate, 0.01% (w/v) bromophenol blue) containing 1% (w/v) dithiothreitol, and for another 15 min in equilibration buffer containing 2.5% (w/v) iodoacetamide. IPG strips were then transferred to 18-cm long, 12% acrylamide gels and separated by electrophoresis with the SE 600 Ruby apparatus (GE Healthcare). Gels were fixed overnight in 30% ethanol, 10% acetic acid, and silver stained with a commercial kit (GE Healthcare).

For visualization and analysis, gels were scanned (Image Scanner; GE Healthcare), processed, and statistically analyzed with the Image Master 2D Platinum 6.0 software (GE Healthcare). Spot discrimination was done with the following parameters: (1) smooth factor: 2; (2) minimal area: 5 pixels; and (3) saliency: 100. Analysis was visually corrected for artifact elimination. For each individual spot, background was subtracted and individual intensity volume was normalized by total intensity volume (all-spot intensity). For comparison of the same spot among gels, a minimum of a twofold intensity difference (with $P < 0.01$ according to Student's *t*-test) was established to consider a differential expression.

Protein identification by mass spectrometry

The spots of interest from 2D separations were cut off the gels, dehydrated in acetonitrile, vacuum evaporated, and resuspended in NH_4HCO_3. The two-dimensional liquid chromatography fractions were also vacuum evaporated and residues re-suspended in NH_4HCO_3. Samples were then reduced with 10 mmol/l dithiothreitol in 50 mmol/l NH_4HCO_3 at 56 °C, and alkylated with iodoacetamide in 50 mmol/l NH_4HCO_3. Proteins were in-gel digested with porcine trypsin (Promega, Madrid, Spain), and peptides were extracted with 0.5% (v/v) trifluoroacetic acid, vacuum evaporated, and redissolved in 0.1% (v/v) formic acid. Peptide-containing solutions were injected in a LC-ESI-QUAD-TOF (liquid chromatography-electrospray ionization-quadrupole time-of-flight) mass spectrophotometer QSTAR XL (Applied Biosystems, Carlsbad, CA) with an 1100 micro HPLC (Agilent, Madrid, Spain). A wide pore 150 × 0.32 mm (5 µm) Supelco column (Discovery BIO, Seattle, WA) was used. Tandem mass spectra were obtained. Protein identification was performed using the MASCOT software (www.matrixscience.com) against non-redundant protein sequence databases (Swiss Prot and National Center for Biotechnology Information). Mass tolerance was set at 50 p.p.m., tandem mass spectra tolerance was 0.5 Da, and the taxonomic status was *Rattus*. Only significant hits, as identified by MASCOT probability analysis, were considered and at least one peptide match with ion score above 20 was set as the threshold of acceptance. For confirmation, some proteins were also identified with an Ultraflex I MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrophotometer (Bruker Daltonics, Bremen, Germany).

Gene expression analysis

Reverse transcriptase-PCR amplification of reg IIIb, gelsolin, and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was performed with the following primers: for rat reg IIIb, 5′-TTGTTTGAT GCAGAACTGGC-3′ and 5′-AGACGTAGGGCAACTTCACT-3; for rat gelsolin, 5′-CAAGGCTACTTCAAGTCTGG-3′ and 5′-GCTACC CTCTTCAGACACAT-3; for rat GAPDH, 5′-GTGGTCATGAGCC CTTCCA-3′ and 5′-AACTCCCTCAAGATTGTCAGCAA-3′. PCR conditions were: $1 \times (95\,^{\circ}C \times 4\,min)$; $30 \times (95\,^{\circ}C \times 1\,min + T_m \times 1\,min)$; and $1 \times (72\,^{\circ}C \times 10\,min)$; where T_m was 55.5 °C for reg IIIb, 55.0 °C for gelsolin, and 55.9 °C for GAPDH. The signal obtained was quantified by densitometry using the Scion Image software.

Excretion studies with *in situ* perfused kidneys

At the end of the treatment, rats treated with gentamicin for 6 days were anesthetized and an extracorporeal circuit for kidney perfusion was set up, as described elsewhere,[69] with some modifications. Briefly, the renal artery, vein, and ureter of the right kidney were ligated. The renal artery and vein of the left kidney and the urinary bladder were cannulated. Oxygenated and warm (37 °C) Krebs-dextran (40 g/l of dextran (molecular weight 64K–76K) in Krebs solution (118.3 mmol/l NaCl, 4.7 mmol/l KCl, 1.8 mmol/l $CaCl_2$, 1.2 mmol/l $MgSO_4$, 1.2 mmol/l KH_2PO_4, 25 mmol/l $NaHCO_3$, 0.026 mmol/l EDTA, and 11.1 mmol/l glucose, pH = 7.4)) was perfused through the renal artery at 3 ml/min, and was discarded through the renal vein. Urine fractions were collected from a catheter placed in the urinary bladder, starting before the perfusion with Krebs (when blood was still passing through the kidney), and for 2 h after perfusion with Krebs started. All urine samples were kept at −80 °C until assayed by western blot for the presence of reg IIIb and gelsolin.

Statistical analysis

Data are represented as the mean ± s.e. of *n* experiments performed, as indicated in each case. Except for the study of proteomic results (as indicated above), statistical comparisons were assessed by one-way analysis of variance.

DISCLOSURE

JML-N and FJL-H are minority shareholders of Bio-inRen, SL, a biotech company holding license on the patent on the use of gelsolin and reg IIIb as markers for the diagnosis of kidney injury. All the other authors declared no competing interests.

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